cAMP/PKA pathway activation in human mesenchymal stem cells *in vitro* results in robust bone formation *in vivo*

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Tissue engineering of large bone defects is approached through implantation of autologous osteogenic cells, generally referred to as multipotent stromal cells or mesenchymal stem cells (MSCs). Animal-derived MSCs successfully bridge large bone defects, but models for ectopic bone formation as well as recent clinical trials demonstrate that bone formation by human MSCs (hMSCs) is inadequate. The expansion phase presents an attractive window to direct hMSCs by pharmacological manipulation, even though no profound effect on bone formation *in vivo* has been described so far using this approach. We report that activation of protein kinase A elicits an immediate response through induction of genes such as *ID2* and *FosB*, followed by sustained secretion of bone-related cytokines such as BMP-2, IGF-1, and IL-11. As a consequence, PKA activation results in robust *in vivo* bone formation by hMSCs derived from orthopedic patients.

bone tissue engineering | osteogenesis | PKA signaling

The ability of human mesenchymal stem cells (hMSCs) to differentiate into adipogenic, chondrogenic, osteogenic (1), and myogenic (2) lineages has generated a great deal of potential clinical use in regenerative medicine and tissue engineering in the past decade. Concomitantly, hMSCs are increasingly used as a cell biological model system to investigate molecular mechanisms governing signal transduction (3), differentiation (4–6), cell fate decision (7), and senescence (8, 9) because the step from basic research to clinical application is relatively short. Although hMSCs are multipotent and form mineralized bone tissue *in vivo*, their clinical application is still limited. Using a bone tissue engineering approach, large bone defects can be repaired in animal models by using animal-derived MSCs. For instance, Petite *et al*. (10) demonstrated bone union in a metatarsal defect in sheep. In contrast, bone formation by hMSCs is substantially less robust, and repair of critical size bone defects has not been achieved by hMSCs so far (11).

Predifferentiation of hMSCs into the osteogenic lineage *in vitro* during the expansion phase before implantation offers an opportunity to improve their *in vivo* performance. In previous studies, dexamethasone (dex) and vitamin D3 were used to promote hMSC differentiation *in vitro* (12). More recent studies include the MAPK pathway (13), Rho kinase (7), Wnt (5), Notch (14), and receptor tyrosine kinases (3). So far, no positive correlation between hMSC osteogenesis *in vitro* and bone formation *in vivo* has been reported. Although many reports describe the positive effect of compounds on osteogenesis *in vitro*, there are, to the best of our knowledge, no convincing reports where *in vitro* manipulation of clinically applicable hMSCs significantly augments bone formation *in vivo*. Either no *in vivo* experiments were performed or the effect on bone formation is marginal and sometimes even negative (15, 16). In other studies, transgenic immortalized hMSC derivative was used (3, 9).

Protein kinase A (PKA) signaling plays a prominent but ambiguous role in mesenchymal cell fate decision, which depends on the molecular and developmental context in which the PKA signal is presented (17, 18). Relatively little is known about the role of PKA in osteogenic differentiation of hMSCs, but it is anticipated by the anabolic effect of certain PKA-activating hormones on bone mineral density. Intermittent administration of parathyroid hormone increases trabecular and cancelleous bone formation in ovariectomized mice, although continuous administration results in net bone loss (19). The effect of PKA activation on osteogenesis has been studied in different cell types with compounds that directly or indirectly activate PKA, although the results are contentious. The most direct evidence on a role of PKA in osteogenic differentiation is from studies in calcifying vascular cells (20). Here activation of the PKA pathway with N^6,2′-*O*-dibutyryladenosine-3′,5′-cyclic monophosphate (db-cAMP) stimulated the expression of osteogenic marker genes and *in vitro* mineralization, suggesting that the PKA pathway promotes vascular calcification by enhancing osteogenic differentiation of calcifying vascular cells. Furthermore, the PKA activator forskolin increased bone nodule formation at low concentrations but inhibited it at higher concentrations (21). A recent study shows that parathyroid hormone-related peptide (PTHrP) inhibits *CBFA1* expression through the PKA pathway (22), and it has been reported that PKA activation enhances adipogenic differentiation of hMSCs (18). In this article we describe that PKA-activated hMSCs demonstrate enhanced *in vitro* osteogenesis and *in vivo* bone formation, which opens a promising window of opportunity to further improve bone tissue engineering protocols.

Results

cAMP/PKA Signaling Induces Osteogenesis in hMSCs. To assess the effect of PKA activation on osteogenesis in hMSCs, we exposed a panel of hMSCs, isolated from the bone marrow of 14 patients undergoing orthopedic surgery ranging 31–82 years of age [supporting information (SI) Table S1], to the PKA activator db-cAMP. Both dex and db-cAMP consistently enhanced the expression of the early osteogenic marker alkaline phosphatase (ALP), ranging from a 1.8-fold increase to a 5.3-fold increase compared with untreated controls (see Fig. 1*a* and Fig. S1*a*). Donor variation was observed both in the basal level of ALP expression, as reported by us previously (23), and in their response to both agents (Fig. S1*a*). db-cAMP-induced ALP expression did not depend on the presence of dex, although coexposure resulted in an additive and sometimes

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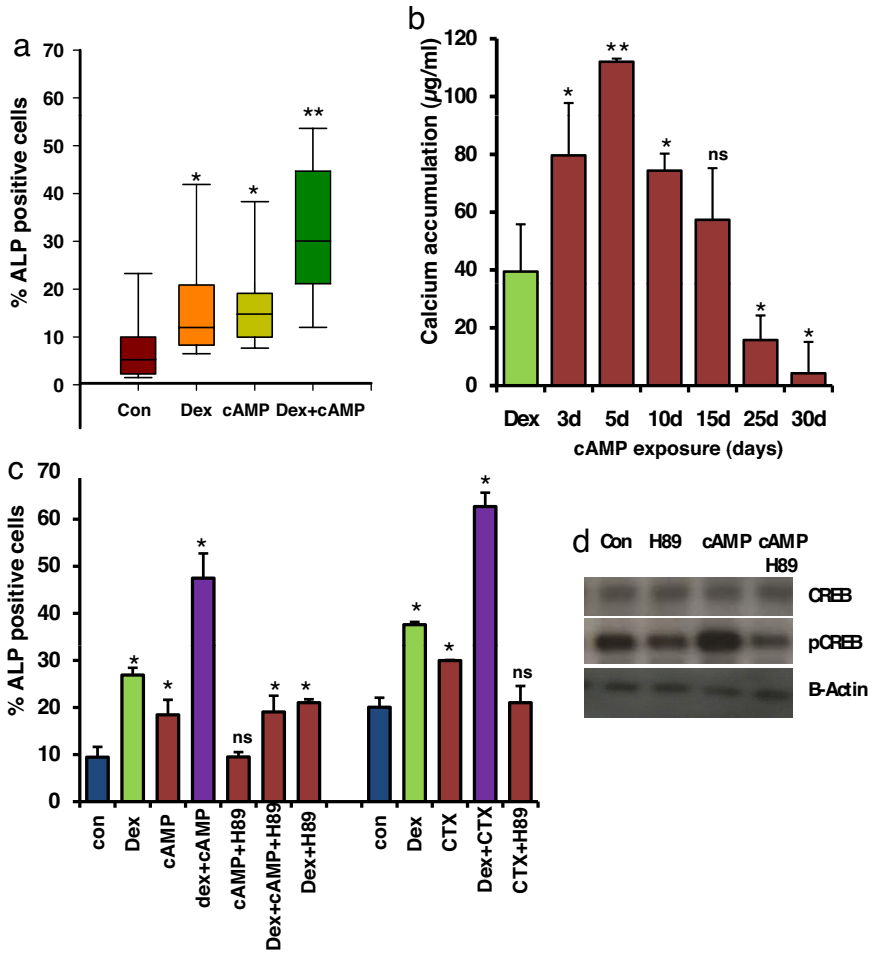

Fig. 1. PKA activation induces *in vitro* osteogenesis of hMSCs. (*a*) Box plot showing the average percentage of ALP-positive cells from 14 donors in basic medium (Con), osteogenic medium (Dex), basic medium with 1 mM db-cAMP (cAMP), or osteogenic medium supplemented with 1 mM db-cAMP (Dex+cAMP). The data were analyzed by using two-way ANOVA followed by Dunnet's multiple-comparison test. Statistical significance is denoted compared with the control group. (*b*) hMSCs were grown in either mineralization medium (dex) or mineralization medium to which 1 mM db-cAMP was added during the first 3, 5, 10, 15, 25, or full 30 days after which calcium deposition was measured and expressed as micrograms of calcium per milliliter of sample. The data were analyzed by using one-way ANOVA followed by Dunnet's multiple-comparison test. (*c*) H89, a PKA inhibitor, reverses the db-cAMP-induced ALP expression. hMSCs were preincubated with H89 for 10–15 h and then cotreated with db-cAMP or cholera toxin (CTX) for 4 days. The data were analyzed by using one-way ANOVA followed by Tukey's multiple-comparison test. (*d*) Addition of db-cAMP to hMSCs for 6 h resulted in increased phosphorylation of transcription factor CREB, which could be inhibited by coincubation with H89. *, $P < 0.05$; **, $P < 0.01$.

synergistic effect on ALP expression, resulting in up to 60% of ALP-positive cells in some donors. Consistent with a positive effect on ALP expression, calcium deposition was enhanced when db-cAMP was administered to hMSCs in mineralization medium (Fig. 1*b*). Optimal stimulation of mineralization was observed when hMSCs were exposed to db-cAMP during the first 5 days of osteogenic culture, resulting in a 3-fold increase in mineralization compared with treatment with dex alone. Incubation for >15 days resulted in a significant inhibition of mineralization. The positive effect of PKA signaling on osteogenesis was further confirmed by quantitative PCR (qPCR) on a panel of osteogenic marker genes during a 15-day osteogenic time course. db-cAMP had a significant positive effect on *ALP* and *COL1A1* expression from day 3 until day 15 (Fig. S2). Dex and db-cAMP together induced the expression of the transcription factor *CBFA1* on days 5 and 15. In contrast, *OPN* expression was induced only at day 10 by dex or dex and db-cAMP and appears to be a dex-mediated event. *BGLAP*, *ON*, and *S100A4* showed no consistent difference in expression profile during the course of osteogenic differentiation.

To demonstrate that db-cAMP mediates its effect through activation of PKA, we exposed cells to two other upstream PKA activators, cholera toxin and forskolin, and confirmed that also these compounds stimulate ALP expression in hMSCs (Fig. 1*c* and Fig. S1*b*). When hMSCs were exposed to db-cAMP or cholera toxin in the presence of H89, an inhibitor of PKA activity, PKA-induced ALP was reduced to basal level. Interestingly, H89 did not significantly affect dex-induced ALP expression, showing that dex and db-cAMP stimulate ALP expression through discrete molecular mechanisms (Fig. 1*c*). One of the direct target proteins of PKA is cAMP response element-binding protein (CREB), and indeed we detected phosphorylated CREB in hMSCs upon treatment with db-cAMP (Fig. 1*d*). Evidently, treatment of hMSCs with db-cAMP leads to PKA activation, which stimulates *in vitro* osteogenic differentiation of hMSCs.

cAMP/PKA Signaling Enhances *in Vivo* Bone Formation by hMSCs. To evaluate the effect of db-cAMP on *in vivo* bone formation we used the ectopic bone formation model in immune-deficient mice, which is widely used to assess the bone-forming capacity of hMSCs (3, 9, 11, 24). Using this model, we typically observe a bone/ceramic surface ratio of 15–20% for goat MSCs, as shown in Fig. 2*a*. This represents ≈50% of the available pore area, and all of the pores

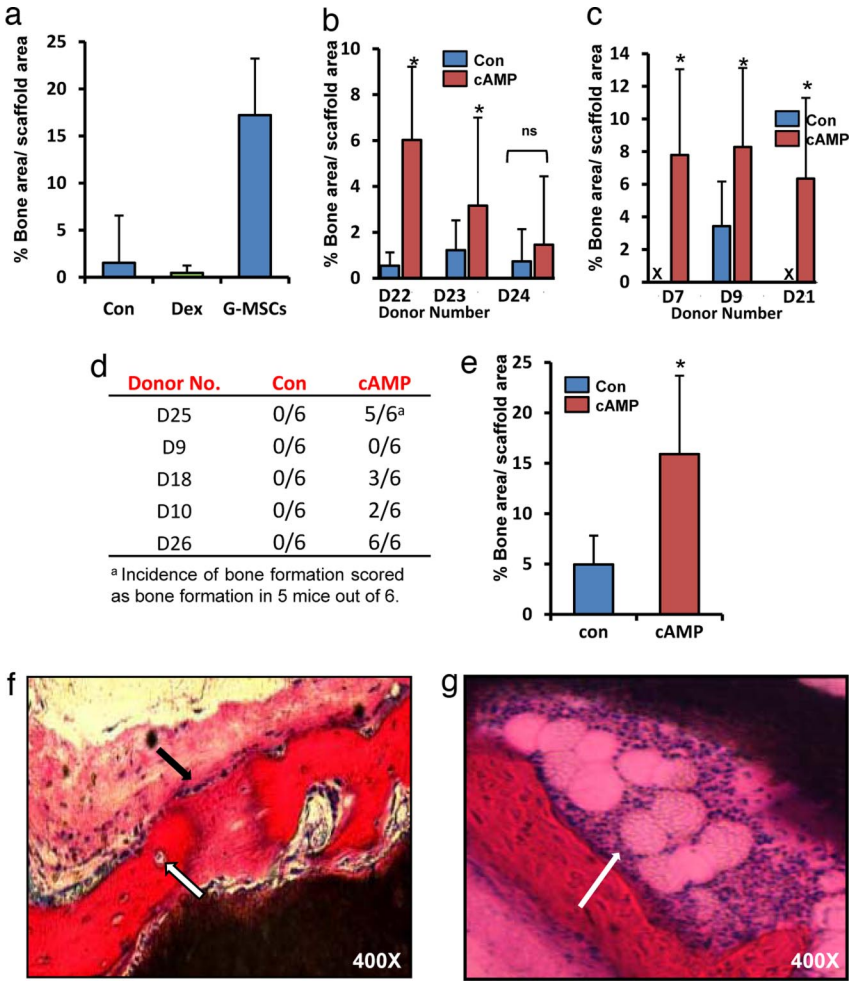

Fig. 2. db-cAMP augments the *in vivo* bone-forming capacity of hMSCs. (*a*) hMSCs were cultured on BCP particles in basic medium (Con) or osteogenic medium (Dex) for 7 days and implanted s.c. in nude mice for 6 weeks. Histomorphometric analysis demonstrates that osteogenic medium does not affect *in vivo* bone formation. Note the amount of bone formed by an equal number of goat-derived MSCs (G-MSCs) in an independent experiment. (*b*) *In vivo* bone formation by hMSCs from three donors using the standard tissue engineering approach (see *Materials and Methods*). ns, not significant. (*c*) Bone formation using the peroperative seeding approach. Note the consistent increase in bone formation upon db-cAMP treatment. The data from *b* and *c* were analyzed by using Student's *t* test compared with their respective controls. (*d*) Incidence of bone formation using the peroperative seeding approach by hMSCs from five donors. (*e*) *In vivo* bone formation by hMSCs cultured in a perfusion bioreactor system in proliferation medium (con) or proliferation medium supplemented with 1 mM db-cAMP (cAMP). The data were analyzed by using Student's *t* test. (*f*) A representative histological section showing newly formed bone (red), matrix-embedded osteocytes (white arrow), and lining osteoblasts (black arrow). (*g*) Bone marrow-like tissue was seen at multiple places in bone derived from db-cAMP-treated hMSCs (white arrow). *, $P < 0.05$.

contain bone tissue. In contrast, 1–2% bone/ceramic surface ratio is observed when hMSCs were grown in the presence or absence of dex (Fig. 2*a*). To assess whether db-cAMP-treated hMSCs display enhanced bone formation, we analyzed ectopic bone formation by hMSCs from 11 donors undergoing orthopedic surgery. First, hMSCs from three donors were seeded onto porous calcium phosphate ceramics and cultured for 7–10 days in basic medium. During the last 4 days, the cells were grown in the presence or absence of 1 mM db-cAMP. *In vivo* bone formation by hMSCs increased from 1.5% in the control group up to 6% upon db-cAMP exposure (Fig. 2*b*). To further investigate the potential application of db-cAMP-treated hMSCs in bone tissue engineering, we tested the effect of db-cAMP in two alternative strategies currently under investigation: peroperative seeding of hMSCs and bioreactor-mediated bone tissue engineering. We expanded hMSCs from eight donors and changed the medium to basic medium or basic medium containing 1 mM db-cAMP 4 days before implantation. On the day of the operation, we trypsinized the cells, allowed them to attach to porous ceramic scaffolds for 4 h, and analyzed bone formation using ectopic implantation model. Surprisingly, we observed no bone formation in the control group except for one donor (Fig. 2 *c* and *d*). Apparently, peroperative seeding represents a so far unreported very stringent tissue engineering protocol. In contrast, bone formation was observed in seven of eight donors analyzed (Fig. 2 *c* and *d*) whereas the bone/ceramic surface ratio increased up to 8% (Fig. 2*c*) upon exposure to db-cAMP. When the data from Fig. 2 *b* and *c* are combined the average bone/scaffold surface ratio significantly increases from 1.0 ± 1.2% by untreated cells to 5.6 ± 2.7% when db-cAMP-treated hMSCs were implanted ($P < 0.01$ using one-way ANOVA).

Next we investigated whether db-cAMP could be implemented in bioreactor-mediated bone tissue engineering. Methylene blue staining of tissue-engineered constructs grown for 7 days in a bioreactor suggested that db-cAMP had a negative effect on cell proliferation (Fig. S3*a*). We confirmed this with hMSCs grown in 2D, where db-cAMP dose-dependently inhibited hMSC proliferation (Fig. S3*b*). Moreover, db-cAMP-treated hMSCs displayed a 17-fold up-regulation of *GAS1* (see Table S2), a gene that is known

to inhibit cell proliferation (25). Despite the negative on proliferation, db-cAMP-treated hMSCs produced three times more bone (15% bone/ceramic surface ratio) than untreated control cells (5%), which is equal to the amount of bone deposited by goat MSCs (Fig. 2*a*). Histological examination of the explanted grafts showed that mature bone was formed in which osteocytes were embedded in mineralizing extracellular matrix (Fig. 2*f*) and mineralizing osteoblasts could be detected. Polarizing light microscopy of the deposited matrix showed areas of lamellar bone, which indicates that bone tissue has been remodeled by osteoclasts and osteoblasts (Fig. S4). Moreover, marrow-like tissue was observed in the vicinity of the tissue-engineered bone, which further indicates the functionality of the bone tissue (Fig. 2*g*) (24).

Autocrine and Paracrine Induction of Bone Formation by cAMP/PKA-Activated hMSCs. Based on the robust effect of db-cAMP on bone formation *in vivo*, which is in striking contrast to the effect of dex, we anticipated that PKA activation results in the expression of a unique set of target genes ultimately resulting in osteogenic differentiation *in vitro* and bone formation *in vivo*. To get more insight into the molecular mechanism behind db-cAMP-induced bone formation, we compared the gene expression profile of untreated cells with that of hMSCs treated with db-cAMP for 6 h using DNA microarray technology. Among the 62 genes up-regulated six times or more by db-cAMP (Table S2), a number of genes were previously associated with cAMP/PKA signaling. Six genes were identified in chip-on-chip assays for CREB-binding promoters (*CREM*, *RCC1*, *GP-1*, *FLRT3*, *GPRC5A*, and *EVX-1*; see http://natural.salk.edu/CREB/) whereas other genes have been implicated in cAMP/PKA signaling, e.g., *ID-2* (26), *BMP-2* (27), *IGF-1* (28), and *IL-11* (29). To show that the genes are directly activated by PKA signaling, we demonstrated that db-cAMP-mediated *ID-2* expression is insensitive to cyclohexamide, an inhibitor of translation (Fig. 3*a*). Gene Ontology enrichment of the data set further showed a remarkably high number of transcription factors (nine of the top 62 up-regulated genes). Three transcription factors are implicated in osteogenesis [*c-Fos*, *FosB*, and *ID-2* (30)], but others were previously not implicated in osteogenesis, such as *BCL3*, *EVX1*, *CDX2*, *DUX1*, *DAT1*, and *ID-4*. We did not observe the classical osteogenic transcription factors such as *CBFA1* (31) and *OSX/SP7* (32) in our microarray data set. We also analyzed the gene expression profile of hMSCs at the time of implantation. Gene Ontology enrichment of genes up-regulated five times or more in hMSCs treated for 7 days with db-cAMP shows that, at this point in time, hMSCs express many secreted proteins, mostly growth factors and cytokines (Table S3). Most striking is the 46-fold up-regulation of *BMP-2*, which suggests that autocrine or paracrine BMP signaling is involved in the osteogenic effect of db-cAMP. To investigate this, we analyzed the expression of *ID-1*, a typical BMP target gene, by qPCR during db-cAMP-driven *in vitro* differentiation. When hMSCs were exposed to db-cAMP and dex, *ID-1* expression was significantly higher on day 5 compared with db-cAMP alone. Dex alone did not affect *ID-1* expression compared with untreated cells (Fig. 3*b*). Other cytokines that have been implicated in osteogenesis and bone formation (*IGF-1*, *IL-8*, and *IL-11*) (33) ranked among the most highly regulated genes (19-, 47-, and 18-fold up-regulated, respectively; see Table S3). We confirmed the secretion of these cytokines by db-cAMP-treated hMSCs using ELISA (Fig. 3*c*). In this case, the induction is even more drastic. Another interesting set of cytokines up-regulated in the 7-day treatment group are proteins interfering with vasculogenesis such as angiopoietin-like 2, angiopoietin-like 4, and placental growth factor. Based on the putative involvement of BMP and other secreted proteins, we propose a model for db-cAMP-induced bone formation in which paracrine signaling through hMSC-secreted cytokines and growth factors stimulates bone formation *in vivo* (Fig. 4).

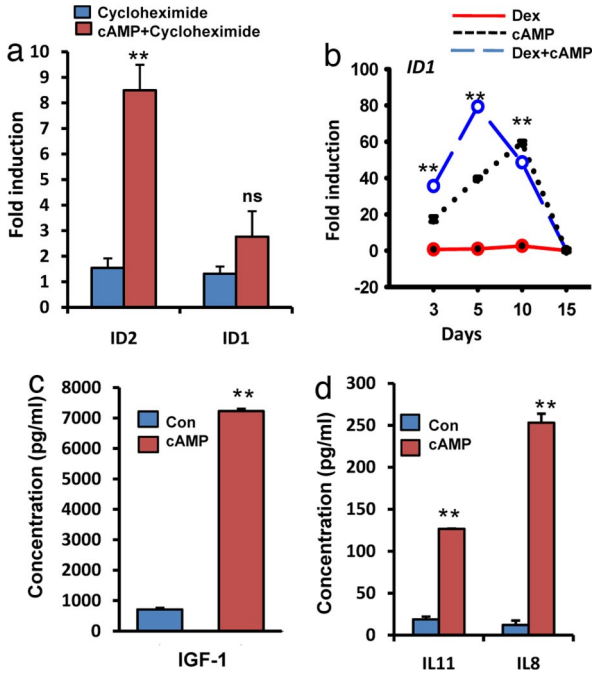

Fig. 3. db-cAMP-induced gene and protein expression. (*a*) hMSCs were treated with cycloheximide for 1 h and then coincubated with db-cAMP for 6 more hours. Expression of BMP target genes *ID-1* and *ID-2* was analyzed compared to cycloheximide-treated cells. The data were analyzed by using Student's *t* test. (*b*) hMSCs were grown in basic medium, basic medium supplemented with 1 mM db-cAMP (cAMP), osteogenic medium (Dex), or osteogenic medium supplemented with 1 mM db-cAMP (Dex+cAMP). Expression of *ID-1* was analyzed by qPCR and is expressed as fold induction compared with cells grown in basic medium. The data were analyzed by using two-way ANOVA. Statistical differences are denoted compared with cells grown in basic medium. (*c* and *d*) db-cAMP induces secretion of proosteogenic cytokines and growth factors. hMSCs were treated with db-cAMP for 4 days, the supernatant was collected, and IGF-1 (*c*), IL-8, and IL-11 (*d*) expression in the medium was measured by ELISA. The data were analyzed by using Student's *t* test. **, $P < 0.01$.

Discussion

The osteogenic potential of hMSCs has been long recognized and provides the exciting potential to treat patients with bone defects beyond spontaneous healing (1, 34). Successful bone formation has been reported for animal models, but so far bone formation by hMSCs is limited and recent clinical trials have demonstrated that *in vivo* bone formed by the implanted hMSCs is insufficient (11). Bone tissue engineering may be augmented through systemic administration of osteogenic agents or by scaffold-mediated delivery of biologically active reagents (35). Manipulation of hMSCs during *in vitro* expansion is an interesting alternative because, in this approach, the agents used *in vitro* can be cleared from the graft before implantation with minimal regulatory and safety implications. As such, large libraries of small molecules can be screened and used for their desired biological effect (36). The lack of irrefutable data demonstrating that predifferentiation of hMSCs *in vitro* results in enhanced bone formation *in vivo* led us to investigate whether db-cAMP treatment enhances *in vivo* performance of hMSCs. Our data describe the osteogenic effect of PKA activation in hMSCs, and we demonstrate that *in vitro* manipulation of hMSCs with the small molecule db-cAMP results in robust osteogenic differentiation *in vitro* and bone formation *in vivo*. The mechanism of action seems to be different from that used by dex and may involve paracrine/autocrine signaling by BMPs and other osteogenic cytokines. However, other mechanisms such as improved survival of db-cAMP-treated cells or enhanced angiogenesis cannot be ex-

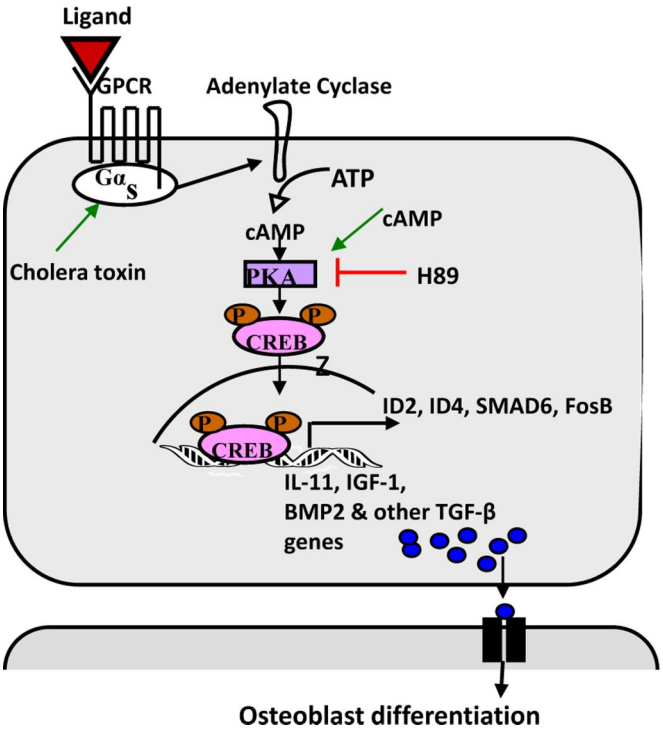

Fig. 4. Model for autocrine/paracrine induction of osteogenesis in hMSCs by PKA signaling. db-cAMP induces direct expression of BMP target genes such as *ID-2* and *ID-4* via CREB resulting in cell-autonomous stimulation of osteogenesis whereas expression of BMP-2, proosteogenic cytokines, and growth factors results in paracrine induction of bone formation.

cluded, which warrants further research to confirm our model (Fig. 4).

We evaluated the potential application of db-cAMP-treated hMSCs in bone tissue engineering by testing its effect on *in vivo* bone formation using different tissue engineering strategies currently in use: standard tissue engineering, peroperative seeding, and bioreactor-based bone tissue engineering. In the peroperative seeding strategy, hMSCs are poli-clinically isolated from a bone marrow biopsy or alternative sources such as fat tissue (37) and expanded. On the day of the surgery, cells are seeded onto the scaffold material and implanted in the defect (38). In addition, automated bioreactor systems may play a role in future bone tissue engineering because a clinical research team can harvest a biopsy and expand and differentiate cell-seeded grafts in routine clinical laboratories up to the time of implantation. Prototypes of bioreactors have been reported by us and others (39, 40). In our hands, bioreactor-mediated bone tissue engineering is the most efficient method. We are trying to enhance bone formation even further by improving the proliferation phase of MSCs before db-cAMP treatment and by finding the most optimal balance between induction of osteogenesis and cell proliferation. With further improvement on the proliferation and differentiation scheme, we hope to consistently achieve a 15–20% bone/ceramic surface ratio.

The data presented in this article describe for the first time that bone formation by hMSCs can be significantly augmented through manipulation of the signal transduction milieu *in vitro* by using a simple compound like db-cAMP. This work once again demonstrates the enormous plasticity of hMSCs and strongly encourages further efforts to engineer the gene expression profile of hMSCs for optimal clinical application.

Materials and Methods

Isolation, Culture, and ALP Analysis of hMSCs. Bone marrow aspirates were obtained from donors with written informed consent, and hMSCs were isolated and proliferated as described previously (41). Briefly, aspirates were resuspended by using 20-gauge needles, plated at a density of 5×10^5 cells per square centimeter and cultured in hMSC proliferation medium containing α-MEM (Life Technologies), 10% FBS (Cambrex), 0.2 mM ascorbic acid (Asap; Life Technologies), 2 mM L-glutamine (Life Technologies), 100 units/ml penicillin (Life Technologies), 10 μg/ml streptomycin (Life Technologies), and 1 ng/ml basic FGF (Instruchemie). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week, and cells were used for further subculturing or cryopreservation. hMSC basic medium/control medium was composed of hMSC proliferative medium without basic FGF, hMSC osteogenic medium was composed of hMSC basic medium supplemented with 10^{-8} M dex (Sigma), and hMSC mineralization medium was composed of basic medium supplemented with 10^{-8} M dex and 0.01 M β-glycerophosphate (Sigma). Donor information is provided in Table S1.

Osteogenic Assay. To determine whether PKA activation elicits an osteogenic response in hMSCs, we exposed them to 1 mM db-cAMP (Sigma) with or without dex for 4 days and analyzed the expression of the osteogenic marker ALP by flow cytometry. hMSCs were seeded at 5,000 cells per square centimeter and allowed to attach for 10–15 h in basic medium, then cells were incubated with 10^{-8} M dex and 1 mM db-cAMP for the denoted time periods. Each experiment was performed in triplicate with a negative control (cells grown in basic medium) and a positive control (cells grown in the presence of dex) and one or more experimental conditions. At the end of the culture period, the cells were trypsinized and incubated for 30 min in block buffer [PBS with 5% BSA (Sigma) and 0.05% NaN_2], then incubated with primary antibody (anti-ALP, B4-78; Developmental Studies Hybridoma Bank, University of Iowa) diluted in wash buffer (PBS with 1% BSA and 0.05% NaN_2) for 30 min or with isotype control antibodies. Cells were then washed three times with wash buffer and incubated with secondary antibody (rabbit anti-mouse IgG phycoerythrin; DAKO) for 30 min. Cells were washed three times and suspended in 250 μl of wash buffer with 10 μl of Viaprobe (Pharmingen) for live/dead cell staining, and only live cells were used for further analysis. The percentage of ALP-positive cells was calculated on a FACSCalibur (Becton Dickinson Immunocytometry Systems). Mineralization was measured quantitatively by using a calcium assay kit (Sigma Diagnostics; 587A) according to the manufacturer's protocol after culturing the cells for the denoted time period with the compounds mentioned.

Gene Expression Analysis by qPCR and Microarray. The effect of db-cAMP on expression of osteogenic marker genes was analyzed by seeding hMSCs at 5,000 cells per square centimeter in T75 flasks supplemented in various medium compositions for 3, 5, 10, and 15 days. To analyze the direct induction of BMP target genes by PKA activation, hMSCs were seeded at 5,000 cells per square centimeter and supplemented with 1 mM db-cAMP for 6 h with or without 10 μM cycloheximide (Sigma). RNA was isolated by using an RNeasy mini kit (Qiagen), and qPCR was performed by using SYBR green (Invitrogen) on a Light Cycler (Roche). Data were analyzed by using Light Cycler software version 3.5.3, using the fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes was calculated relative to 18s rRNA levels by the comparative ΔCT method (42). To study the genome-wide effect of db-cAMP, hMSCs were grown in either basic medium or basic medium supplemented with 1 mM db-cAMP for 6 h or 7 days. RNA was isolated by using an RNeasy midi kit (Qiagen), and 8 μg of total RNA was used for probe labeling according to the manufacturer's protocol (Affymetrix). The probe quality was verified by using lab-on-chip technology (Agilent Technologies), and samples were hybridized to Human Genome Focus arrays according to the manufacturer's protocol (Affymetrix). Data analysis was performed by using Affymetrix GENECHIP 4.0 software. CREB (Calbiochem), phosphorylated CREB (R & D Systems), and β-actin (R & D Systems) antibodies were used to detect respective proteins by Western blotting on cell lysates obtained from hMSCs treated with various supplements. IGF-1, IL-8, and IL-11 secretion upon db-cAMP treatment was measured in the cell supernatant by ELISA (R & D Systems) according to the manufacturer's protocol.

***In Vivo* Evaluation Studies.** To evaluate the effect of PKA activation on ectopic bone formation by hMSCs, we used three tissue engineering protocols.

Standard tissue engineering approach. As scaffold for all bone tissue engineering methods, we used porous biphasic calcium phosphate (BCP) ceramic granules of ≈2–3 mm, prepared and sintered at 1,150°C as described previously (43). The cells were cultured for 7 days in basic medium, in osteogenic medium, or in osteogenic medium supplemented with 1 mM db-cAMP during the last 4 days. Goat MSCs were isolated and expanded as described previously (40).

Peroperative seeding approach. To investigate the performance of db-cAMP-treated hMSCs in the peroperative seeding approach, we expanded hMSCs from eight donors and changed the medium to basic medium or medium containing 1 mM db-cAMP 4 days before implantation. On the day of the operation, we

trypsinized the cells and allowed them to attach to porous BCP scaffolds for 4 h as described above.

Bioreactor-based tissue engineering. hMSCs were seeded onto porous ceramics as described above, transferred into a bioreactor, and cultured for 4 days in basic medium. A direct perfusion flow bioreactor was used as described previously (40). Briefly, the bioreactor comprised an inner and outer housing, which are configured as coaxially disposed, nested cylinders. The bioreactor system consisted of a bioreactor, a sterile fluid pathway (made of γ-sterilized PVC tubing with low gas permeability) that includes a medium supply vessel, a pump, an oxygenator, and a waste vessel. The fluid pathway contained a temperature sensor and two dissolved oxygen sensors, which are placed at the medium inlet and outlet of the bioreactor. The whole bioreactor system was placed in a temperature-controlled unit at 37°C. The incubation units were equipped with controlled oxygen and carbon dioxide supplying systems. The gas environment in the chamber is kept at a constant level of 21% O_2 and 5% CO_2, and medium is pumped through the gas-permeable tube. This system enables a medium flow over and through the cell-seeded biomaterials with constant pH and a constant oxygen concentration. During the last 4 days, the cells in the bioreactor were or were not supplemented with 1 mM db-cAMP perfused through the medium.

At the end of the culture period, the tissue-engineered constructs were implanted s.c. in immune-deficient mice for 6 weeks. In each *in vivo* experiment, 10 nude male mice (Hsd-cpb:NMRI-nu; Harlan) were anesthetized by intramuscular injection of 0.05 ml of anesthetic (1.75 ml of 100 μg/ml ketamine, 1.5 ml of 20 mg/ml xylazine, and 0.5 ml of 0.5 mg/ml atropine). Four s.c. pockets were made, and each pocket was implanted with three particles of each condition. The incisions were closed with a vicryl 5-0 suture, and the tissue-engineered constructs were left for 6 weeks. All experiments were approved by the local Animal Experimental Committee.

Histology and Histomorphometry. After 6 weeks, the mice were killed by using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck) in 0.14 M cacodylic acid (Fluka) buffer (pH 7.3), dehydrated, and embedded in methyl methacrylate (Sigma) for sectioning. Approximately 10-μm-thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). The sections were stained with basic fuchsin and methylene blue to visualize new bone formation. The newly formed mineralized bone stains red with basic fuchsin, all other cellular tissues stain light blue with methylene blue, and the ceramic material remains black and unstained by both the dyes. Histological sections were qualitatively analyzed by using a light microscope (Leica), and each histological section was scored either positive or negative for bone formation. Quantitative histomorphometry was performed as described previously (44). Briefly, high-resolution digital photographs (300 dpi) were made from four randomly selected sections from each tissue-engineered graft. Before histomorphometrical analysis, bone and material were pseudocolored green and red, respectively, by using Photoshop CS2 (Adobe Systems). Image analysis was performed by using a PC-based system with KS400 software (version 3, Zeiss). A custom-made macro was used to measure bone/ceramic surface ratios.

Cell distribution on the scaffold and matrix formation were qualitatively visualized with methylene blue staining. The tissue-engineered constructs were immersed in 1% methylene blue solution for 1 min and washed three times with demineralized water to remove unbound stain. The BCP particles remain unstained while cells stain blue when examined by light microscopy. To assess the effect of db-cAMP on proliferation, cells were seeded in basic medium at 5,000 cells per square centimeter either with or without 1 mM db-cAMP for 5 days. Next, the medium was removed and 2 ml of a 10% Alamar blue (Biosource) solution was added and incubated for 4 h. From this, 0.2 ml was transferred to a 96-well plate and measured on a spectrophotometer (PerkinElmer) at 545 nm.

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